THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 27, Issue of July 5, pp. 24057–24066, 2002

Printed in U.S.A.

Mer Receptor Tyrosine Kinase Signaling

PREVENTION OF APOPTOSIS AND ALTERATION OF CYTOSKELETAL ARCHITECTURE WITHOUT STIMULATION OR PROLIFERATION*

Received for publication, December 18, 2001, and in revised form, March 22, 2002
Published, JBC Papers in Press, April 2, 2002, DOI 10.1074/jbc.M112086200

Katherine L. Guttridge‡, J. Christopher Luft‡, Thomas L. Dawson‡§, Eva Kozlowska¶, Nupam P. Mahajan¶, Brian Varnum, and H. Shelton Earp¶‖

From the ¶Lineberger Comprehensive Cancer Center, Department of Medicine and Pharmacology, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27599 and §Procter and Gamble Corporation, Cincinnati, Ohio 45202, and Amgen Corporation, Thousand Oaks, California 91320

***Mer* is a member of the *Axl/Mer/Tyro3* receptor tyrosine kinase family, a family whose physiological function is not well defined. We constructed a *Mer* chimera using the epidermal growth factor receptor (EGFR) extracellular and transmembrane domains and the *Mer* cytoplasmic domain. Stable transfection of the *Mer* chimera into interleukin 3 (IL-3)-dependent murine 32D cells resulted in ligand-activable surface receptor that tyrosine autophosphorylated, stimulated intracellular signaling, and dramatically reduced apoptosis initiated by IL-3 withdrawal. However, unlike multiple other ectopically expressed receptor tyrosine kinases including full-length EGFR or an EGFR/*Axl* chimera, the *Mer* chimera did not stimulate proliferation. Moreover, and in contrast to EGFR, *Mer* chimera activation induced adherence and cell flattening in the normally suspension-growing 32D cells. The *Mer* chimera signal also blocked IL-3-dependent proliferation leading to G_1/S arrest, dephosphorylation of retinoblastoma protein, and elongation of cellular processes. Unlike other agonists that lead to a slow (4–8 days) ligand-dependent differentiation of 32D cells, the combined *Mer* and IL-3 signal resulted in differentiated morphology and growth cessation in the first 24 h. Thus the *Mer* chimera blocks apoptosis without stimulating growth and produces cytoskeletal alterations; this outcome is clearly separable from the proliferative signal produced by most receptor tyrosine kinases.**

Mer, a then novel receptor tyrosine kinase, was identified by bacterial phosphotyrosine (Tyr(P)) expression cloning (1). Ling and Kung (2) independently cloned this receptor and referred to it as Nyk. By sequence, *Mer* is the closest mammalian gene to the chicken proto-oncogene, c-*EYK*, identified by Jia *et al.* (3, 4) as the cellular homologue of a chicken retroviral oncogene v-*EYK*. The extracellular domain structural motifs of Mer place it within the Axl/Ark/UFO family of receptor tyrosine kinases (5–7). In addition to Axl and Mer, this family contains, at least, one other member Tyro3 (8) (also named SKY (9), RSE (10), BRT (11), DTK (12), and TIF (13)) and its potential chicken homologue, REK (14). *Mer* mRNA is most highly expressed in testis, ovary, prostate, lung, and kidney and is detected in peripheral blood monocytes but not in granulocytes (1). It is expressed early during mouse embryonic development, being present in the blastocyst (15). Interestingly, despite the fact that *Mer* mRNA is readily detected in neoplastic B- and T-cell lines (1) and is present in childhood acute lymphoid leukemia samples,[1] it is not expressed in normal B- or T-cells even when they are forced to proliferate (15). To date, evidence gathered by multiple groups hints at physiologic roles for the individual family members, but a coherent view of Mer (or Axl or Tyro3) signaling as it relates to function has yet to emerge.

A large scale biochemical purification approach, using conditioned media from cell lines, identified Gas6, a transcript whose expression increases in senescent cells as a ligand for Axl (17). Gas6 also binds to Tyro3 as does protein S (18). Gas6 binds to Axl and Tyro3, with nanomolar affinity (0.4 and 2.9 nM, respectively (19–21)), as well as to Mer (21) although the affinity is low enough, 29 nM, to raise questions as to whether it is a physiologic or sole ligand for Mer.

Axl/Mer/Tyro3 are ectopically or overexpressed in a variety of human tumor cells (15, 22–24), and Axl and Tyro3 when substantially overexpressed in fibroblasts can transform these cells in the absence of ligand (6, 14, 24). Axl signaling was studied in 32D cells, an IL-3[2]-dependent murine leukemic cell line (25), by constructing an EGFR extracellular and transmembrane domain-*Axl* cytoplasmic domain chimera (26). Upon IL-3 withdrawal, ligand-dependent activation of the *Axl* chimera prevented apoptosis and caused proliferation (26). In fibroblasts grown in serum, full-length Axl when activated by Gas6 stimulated growth. Gas6 Axl signaling prevented apoptosis without stimulating growth when cells were challenged by serum starvation, Myc overexpression, or tumor necrosis factor-α (27, 28). Axl, like platelet-derived growth factor, can increase smooth muscle cell motility (29).

Turning to genetic approaches for clues to the function of this family, our group and two others produced gene-targeted mice, deleting *Mer* (30), *Axl* (31), and *Tyro3* (31), respectively. The subtle nature of the individual gene knockout phenotypes,

* This work was supported by NCI Grants CA-68346 and CA-49240 from the National Institutes of Health (to H. S. E.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ These authors contributed equally to this work.
‖ To whom correspondence should be addressed. Fax: 919-966-3015; E-mail: hse@med.unc.edu.

[1] K. L. Guttridge, J. C. Luft, T. L. Dawson, E. Kozlowska, N. P. Mahajan, B. Varnum, and H. S. Earp, unpublished results.

[2] The abbreviations used are: IL-3, interleukin 3; EGF, epidermal growth factor; EGFR, EGR receptor; EMC, *Mer* chimera; Fn, fibronectin; DMEM-H, in Dulbecco's modified Eagle's medium-high glucose; Rb, retinoblastoma protein; FBS, fetal bovine serum; PS, phosphatidylserine; PI, phosphatidylinositol; ERK, extracellular signal-regulated kinase; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; FACS, fluorescence-activated cell sorter; G-CSF, granulocyte colony stimulating factor; IGF, insulin-like growth factor; JNK, c-Jun N-terminal kinase.

monocyte hyper-reactivity to stimuli, and splenic enlargement in part due to apoptotic debris (*Mer* (30)) and being prone to seizures (*Tyro3* (31)) led to a collaboration in which animals lacking all three members of the family were produced. These "triple knockout" animals were infertile, a phenotype due to a Sertoli cell defect (30).

We have shown recently (32) that the apoptotic debris in *Mer*−/− animals and immune dysregulation characterized by high levels of autoantibodies may be the result of a demonstrable defect in triggering the ingestion of apoptotic cellular material. This defect, particularly the immune dysregulation, is even greater in the triple knockout animals (33). A *mer*-specific, selective phagocytic defect toward apoptotic material is also exhibited in the pigmented retinal epithelial cells of the eye (34, 35), leading to retinal degeneration. Investigation of a rat model of adult blindness, in which pigmented retinal epithelial cells fail to ingest the apoptotic tips of rods and cones on an ongoing basis, first identified an inherited mutation in the rat *Mer* tyrosine kinase gene that truncates the gene product (34). Not surprisingly, the *Mer*−/− mice show retinal degeneration as they age.[1] Finally, at least three human families with inherited retinitis pigmentosa have different mutations in the *Mer* TK gene, all of which abolish *Mer* tyrosine kinase activity (35).

To study the signal emanating from the Mer tyrosine kinase, we constructed a chimeric receptor molecule, hoping to achieve stable expression of a ligand-dependent Mer tyrosine kinase. This was accomplished by replacing the extracellular domain of Mer with a ligand binding and transmembrane domain of the rat EGF receptor (EMC). Several groups (36–39) had previously transfected chimera receptor tyrosine kinases into the IL-3-dependent murine leukemic cell line 32D c13 (32D) and had shown that these receptors could replace the ability of IL-3 to suppress apoptosis. All tyrosine kinase transmembrane receptors, except one, resulted in both survival and rapid proliferation of 32D cells even after IL-3 withdrawal. The exception, the IGF_1 receptor, resulted in apoptosis suppression and slow growth in 32D cells (40). The IGF_1 receptor signal resulted in a differentiated granulocyte-type phenotype over 6–8 days, mimicking the effect of a cytokine, G-CSF (41, 42).

Separate populations of 32D cells, which express neither Mer nor EGF receptor, were transfected with either EMC or full-length EGFR, and stable cell populations were selected. Interestingly and in contrast to multiple tyrosine kinases tested in the 32D cell including the EGFR/*Axl* chimera, ligand-dependent EMC activation prevented apoptosis upon IL-3 withdrawal without stimulating proliferation and caused cell shape changes. More surprisingly, in some 32D cell clones the Mer signal produced a dramatic morphologic change that was coincident with a blockade of IL-3-dependent growth. Thus Mer, at least as a chimeric receptor, is capable of producing an unusual signal in comparison to other receptor tyrosine kinases; blocking apoptosis in the absence of increased cell proliferation although altering the cytoskeleton. This latter capability may explain the failure of monocytes (32) and pigmented retinal epithelial cells (34, 35) to trigger the local cytoskeletal changes necessary to ingest apoptotic material in the absence of Mer tyrosine kinase activity.

MATERIALS AND METHODS

Reagents—Murine EGF (receptor grade) was purchased from BD PharMingen. Interleukin-3 (IL-3) was obtained by culturing WEHI cells in Dulbecco's modified Eagle's medium-high glucose (Invitrogen) supplemented with 10% fetal bovine serum (FBS). The monoclonal antibodies specific for phosphotyrosine (PT66 and RC20) were obtained from Sigma and Transduction Laboratories. The anti-rat EGFR antibody used for immunoprecipitation (1382) was made as described using purified rat liver EGFR as an immunogen (43). EGFR immunoblotting was performed using an antibody (number 22) raised against a fusion protein glutathione *S*-transferase/EGFR C terminus (44). Anti-*Mer* antibodies were obtained by immunizing New Zealand White rabbits with a bacterially expressed glutathione *S*-transferase fusion protein containing the C-terminal-most 100 amino acids of human Mer. Anti-*AKT*, anti-phospho-*AKT*, anti-p38, anti-phospho-p38, anti-p44/42, and anti-phospho-p44/42 were obtained from Cell Signaling. Anti-Rb antibody was obtained from BD PharMingen. PI 3-kinase inhibitor LY294 was purchased from Sigma, the MEK inhibitor, U0126, was purchased from Promega.

Engineering of the EGFR/Mer Chimera—The EGFR/*Mer* chimera (EMC) was constructed by combining the extracellular and transmembrane regions of the EGFR with the intracellular domain of Mer. This was accomplished by PCR-directed insertion of a *Sal*I restriction site into a basic stretch of amino acids (RRR or RKR) that are conserved just inside of the transmembrane domain in many tyrosine kinase receptors, including the EGFR and Mer. The EGFR extracellular domain was amplified with oligonucleotide EMC-2R (5′-gagagagagtcgacgcatg aagaggccgagccc-3′) in conjunction with an extended T7 vector primer (attgtaatacgactcactata), generating the EGFR extracellular domain with a silent mutation in the juxtamembrane Arg-Arg-Arg sequence. The PCR product was then cut with *Eco*RI and *Sal*I and cloned into pBSII SK+ (Stratagene). The Mer intracellular domain was amplified with the oligonucleotide EMC-1F (5′-gagagagagagacgtcgacgagtccaggagacaaagtttggg-3′) in conjunction with the extended T7 primer, and the PCR product was TA-cloned (Invitrogen) as per manufacturer's instructions. An *Nsi*I to *Hin*dIII fragment from the original full-length cDNA was used to replace all of the PCR-generated sequence except for the 28 bp immediately downstream of the *Sal*I site. The mammalian expression plasmid was generated by introduction of the full-length chimera *Eco*RI fragment into the *Eco*RI site of the expression vector pLXSN (45).

Construction of Stable 32D Cell EMC and Kinase-inactive EMC Cell Lines—32D parental cells (25) were grown in RPMI 1640 containing 15% heat-inactivated FBS, 5% WEHI 3B conditioned media (to provide IL-3), and 1× penicillin/streptomycin. Cells were washed twice in serum-free RPMI 1640, and then DNA was added, either vector, full-length EGFR, or EMC. The cells were electroporated using the Bio-Rad Gene Pulser Apparatus and immediately transferred to complete media. After 48 h 650 ng/ml geneticin (G418, Invitrogen) was added to the media. The growth and apoptosis analysis experiments shown in this report were replicated using two separate transfection populations and four distinct clones, two from each of the independent populations. Kinase-inactive EMC was made by site-directed mutation of lysine 619 to methionine. The mutated EMC was packaged in a murine retrovirus; 32D cells were infected, and a stable population was selected in G418.

Reverse Transcriptase-PCR Analysis of EMC Expression—Approximately 5×10^6 cells from each cell line were resuspended in 0.5 ml of lysis solution (4 M guanidinium isothiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, and 0.1 M 2-mercaptoethanol), and total RNA was isolated by the acid phenol procedure (46). The first strand cDNA was prepared using Moloney murine leukemia virus reverse transcriptase. Five microliters of the first strand cDNA was amplified in a 50-μl PCR using *Taq* DNA polymerase (Invitrogen). To test for the integrity of the RNA samples and for template standardization, amplification by PCR using actin primers (HACA-1F and HACA-1R) was performed. The expression of *Mer*, EGFR, and EMC in the samples was analyzed using primers specific for each cDNA. Amplifications were performed for 35 cycles with an annealing temperature of 60 °C. The sequence of the primers is as follows: actin-HACA–1F, 5′-CCTTCCTGGGCATGGAGTCCT-3′; HACA-1R, 5′-GGAGCAATGATCTTGATCTTC-3′; *Mer*-3F, 5′-CACCTCTGCCTTACCACATCT-3′; and 2R, 5′-ATCCACAAAAGCAGCAAAGA-3′ (specific to the *mer* extracellular region absent from the chimeric cDNA); EGFR-1F, 5′-AAAGACTGCAAGGCCGTGAA-3′; 2R, 5′-GCCCAGATGGCCACACTTC-3′; EMC–1F, 5′-TAACGTGTGCCACCTCTGC-3′; and 2R, 5′-TCCTCACTGACTCCCAAGC-3′. The PCR products were analyzed by electrophoresis of 10 μl of each reaction on a 1.0 or 1.5% agarose gel containing ethidium bromide.

Cell Proliferation Assay—32D vector alone (control), EGFR, and EMC were seeded at 20×10^4 cells per ml in RPMI 1640 supplemented with 15% heat-inactivated FBS with or without 5% WEHI conditioned medium (source of IL-3) or 100 ng/ml EGF. At the indicated times flasks were scraped, and cells were counted using a hemocytometer.

Western Blot Analysis of Stable Cell Lines—The EMC-expressing cell populations were resuspended at 5×10^5 cells/ml in serum/WEHI-free media and incubated at 37 °C for 1 h prior to each experiment. The cells were then treated with 100 ng/ml EGF, pelleted, and lysed with ice-cold new lysis buffer (NLB, 20 mM HEPES, pH 7.3, 50 mM NaF, 10%

glycerol, 1% Triton X-100, 1 mM Na_3VO_4, 500 mM NaCl, 5 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 1 μl of leupeptin per ml, 4 KIU of aprotinin per ml). Lysates were clarified, divided, and either boiled in SDS Laemmli sample for lysate Westerns or used for immunoprecipitations. Polyacrylamide gels were loaded and immunoblotted.

FACS Analysis of Receptor Expression in 32D Cells—Cells were washed twice with 1× PBS, resuspended in anti-EGFR 1382 polyclonal antibody, and incubated on ice for 30 min. Cells were then washed three times in 1× PBS, resuspended in anti-rabbit IgG conjugated with FITC antibody, and incubated 30 min on ice, followed by 3 washes in 1× PBS and then analyzed by flow cytometry with FACSTAR plus (BD PharMingen).

FACS Analysis of Apoptosis and Cell Cycle—An annexin V-FITC-labeled apoptosis detection kit was used as instructed by the manufacturer (Genzyme). The procedure evaluates an early event in apoptosis, in which phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external environment. Annexin V has a high affinity for PS and therefore binds to cells with exposed PS. Briefly, 32D vector alone or 32D EMC cells were cultured in RPMI 1640 plus 15% heat-inactivated FBS supplemented with no addition, 5% WEHI-conditioned medium (source for IL-3), or 50 ng/ml murine natural EGF (receptor grade) for 24, 48, or 72 h. Both adherent and non-adherent cells were harvested and washed twice in 1× phosphate-buffered saline (PBS). To analyze apoptosis, cells were then resuspended in annexin binding buffer at a concentration of 5×10^5 cells/ml and incubated in the presence of optimized amounts of FITC-conjugated annexin V and propidium iodine (PI) for 15 min. The percentage of apoptotic cells was evaluated by flow cytometry with a FACStar plus (BD PharMingen). To analyze the cell cycle, cells at the indicated times were fixed in ice-cold 70% ethanol overnight. The next day the cells were washed twice in calcium- and magnesium-free PBS, followed by a 1-h incubation with 50 μg/ml PI and 50 μg of DNase-free RNase, and analyzed by flow cytometry.

Cell Staining—32D cells were grown on coverslips in regular media with 100 ng/ml EGF, 10 μM LY294, or 10 μM U0126. At the appropriate time, cells were washed with 1× PBS and then fixed with methanol. Cells were then stained with Wright Giemsa Stain (Sigma), washed with 1× PBS and then briefly with water. Cells were viewed through a ×20 objective, and images were captured through a CCD camera and analyzed by Scion/Photoshop imaging software.

RESULTS

Activity and Stable Expression of EMC—To investigate the consequences of ligand-dependent Mer activation, we constructed a chimeric receptor placing the Mer tyrosine kinase domain under the control of the EGF receptor ligand-binding domain (Fig. 1*A*). The full-length receptor chimera is predicted to be a protein 1143 amino acids in length, with a molecular mass of 127 kDa. However, since the extracellular domain of the EGFR is heavily glycosylated and contributes ~110 kDa to the mature chimera, the estimated molecular mass of the mature EMC is ~170 kDa. Transient transfection of the full-length EGFR and EMC in CHO-K1 cells revealed tyrosine-phosphorylated receptors of the appropriate size on phosphotyrosine immunoblots. In addition, immunoprecipitation with anti-extracellular domain EGFR antibody 1382 precipitated active tyrosine kinases from EGF receptor or EMC-transfected CHO-K1 cells as assessed with *in vitro* autokinase activity assays with added [^{32}P]ATP (data not shown).

Following transfection of the vector alone (pLXSN), full-length EGFR, or EMC and selection in G418, expression of EGFR and EMC mRNA was assessed by reverse transcriptase-PCR. *Mer* mRNA expression from murine spleen is shown for comparison. Murine Mer was not expressed in any 32D cell populations, and the EGF receptor was not detected except in the EGF receptor-transfected line. EMC was expressed only in the lines transfected with EMC cDNA (Fig. 1*B*). Oligonucleotides utilized to detect EMC expression were specifically designed to span the junction between chimeric partners. Likewise, EGFR and Mer expression were verified by utilizing oligonucleotides designed to regions of mRNA not expressed in the chimera (see "Materials and Methods"). To detect surface receptor protein expression, flow cytometry was performed using an antibody that recognized the extracellular domain of the EGFR, the extracellular portion of both EGFR and EMC. Control 32D cells did not express either receptor, whereas the amount of EGFR and EMC on the respective transfected clones was similar (Fig. 1*C*).

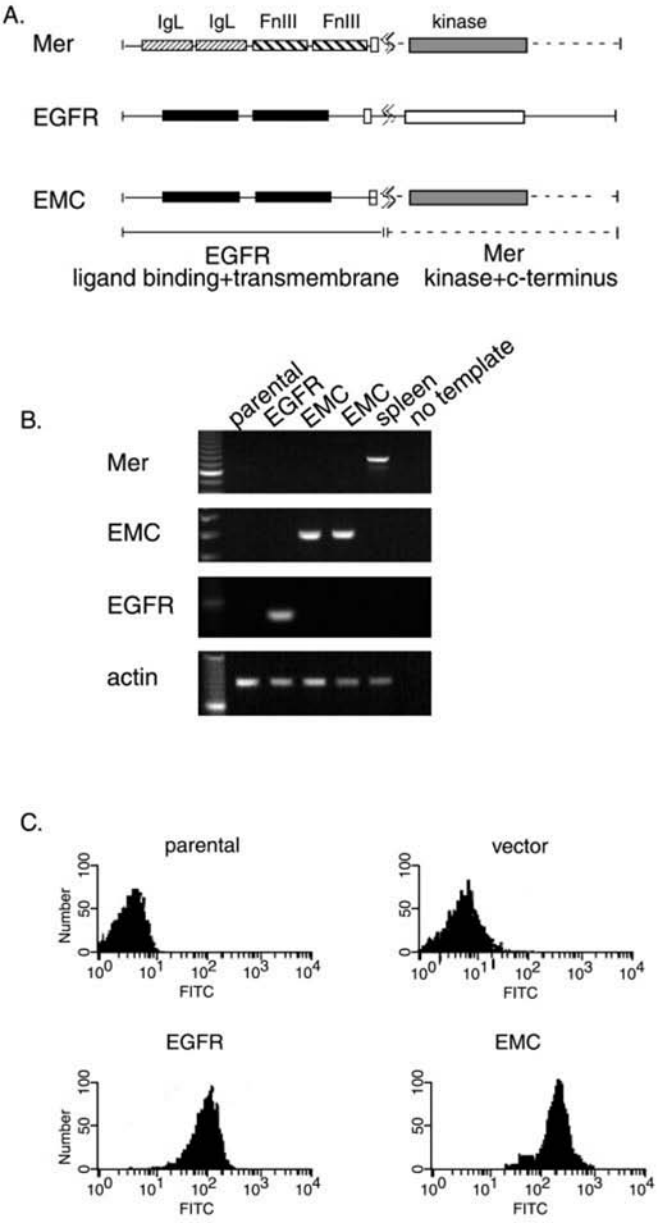

FIG. 1. **Engineering and expression of the EGFR/*Mer* chimera.** *A*, schematic diagrams of the *Mer*, EGFR, and the chimeric receptor (EMC). The *hatched rectangles* represent the immunoglobulin light chain-like domains (*IgL*), fibronectin III-like domains (*FnIII*), or cysteine-rich domains (*black rectangles*) characteristic of the extracellular domains of Mer or EGFR. *B*, analysis of stable expression of the EMC receptor or full-length rat EGFR in 32D cells. Reverse transcriptase-PCR was utilized to confirm expression of transfected receptors and the absence of full-length *Mer* or EGFR in 32D cells. RNA was isolated from parental 32D cells (*1st lane*), and cells transfected with the EGFR (*2nd lane*), EMC (*3rd* and *4th lanes*, two separate populations of transfected 32D cells). RNA from mouse spleen was also isolated and subjected to PCR (*5th lane*). *C*, cycling 32D parental, vector, EGFR, and EMC cells were incubated with rabbit anti-rat extracellular EGFR antisera 1382, followed by incubation with FITC-labeled and anti-rabbit IgG and subjected to analysis by flow cytometry.

Ligand-dependent Signaling by the EGFR, Mer Chimeric Receptor—Neither EGFR nor EMC were expressed at levels that produced constitutive, ligand-independent receptor tyro-

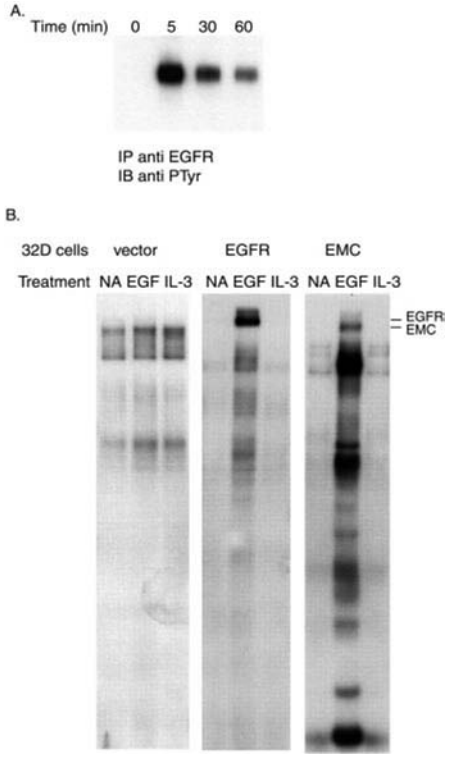

FIG. 2. **Ligand-induced tyrosine phosphorylation in 32D cells.** Comparison of phosphotyrosine substrates from EMC, EGFR, or vector expression cells. *A*, 32D EMC cells were stimulated with 100 ng/ml EGF and lysed 0, 5, 30, or 60 min later. EMC was immunoprecipitated (*IP*) with anti-EGFR antibody 1382 followed by gel electrophoresis and immunoblotting (*IB*) with anti-phosphotyrosine monoclonal antibody (RC20). *B*, vector, EGFR, EMC 32D cells were left unstimulated (no addition, *NA*) or stimulated with 100 ng/ml or IL-3 (5% WEHI conditioned medium) for 10 min. Cell lysates were immunoprecipitated with anti-phosphotyrosine monoclonal antibody (PT66), and precipitates were subjected to gel electrophoresis and immunoblotting with an anti-phosphotyrosine monoclonal antibody (RC20).

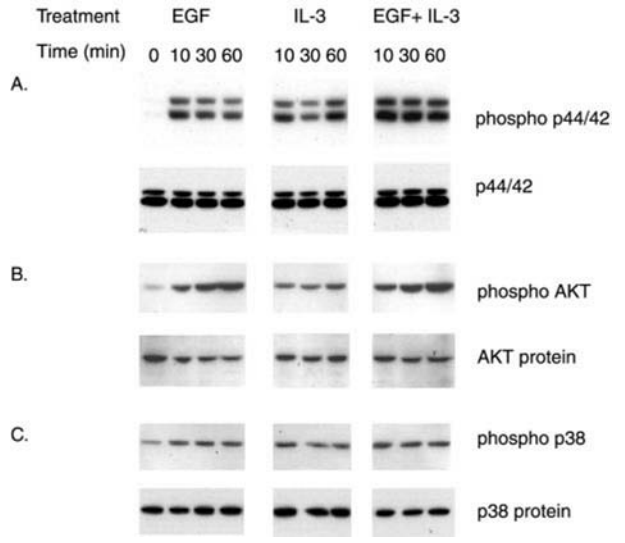

FIG. 3. **EMC activates ERK, *AKT*, and p38 mitogen-activated protein kinase.** IL-3 was withdrawn from cells for 1 h followed by stimulation with 100 ng/ml EGF, IL-3 (5% WEHI conditioned medium), or both EGF and IL-3. Cells were lysed at indicated times, and lysates were subjected to gel electrophoresis and immunoblotted with anti-phospho ERK and anti-ERK (*A*); anti-phospho-*AKT* and anti-*AKT* (*B*); and anti-phospho-p38 and anti-p38 antibody (*C*).

sine phosphorylation. Fig. 2*A* shows the absence of EMC tyrosine phosphorylation in untreated cells and the rapid accumulation of tyrosine phosphate in EMC immunoprecipitated from EGF-treated cells. Other experiments showed EMC tyrosine phosphorylation within 60 s of EGF addition. The results indicate that EMC-expressing 32D cells are an excellent model in which to assess ligand-dependent Mer signaling. To study whether the EGF receptor or the *Mer* chimera activated downstream events differentially, we examined phosphotyrosine (Tyr(P)) containing substrates after a 5-min EGF stimulation of full-length EGFR or EMC-expressing 32D cell lines. EGF stimulated tyrosine phosphorylation of multiple substrates in both cell lines; the pattern of Tyr(P) substrates clearly differed between the two receptors. As expected, EGF did *not* stimulate tyrosine phosphorylation in parental or vector-transfected 32D cells. The similarity of ligand-dependent receptor autophosphorylation in the EGF and EMC-expressing 32D cell lines substantiates the comparable expression of receptor as indicated by flow cytometry (Fig. 2). The pattern of tyrosine-phosphorylated substrates in EMC-bearing cells was both more substantial and complex than that of the EGFR-bearing cells. Thus, despite similar receptor levels on the two cell lines, the differences in tyrosine phosphorylation, in some way, engendered the distinct biologic actions elicited by Mer and EGFR signaling (see below).

To determine the downstream signaling pathways stimulated by EMC, we assessed the activation of members of the mitogen-activated protein kinase family, ERKs 1 and 2 (p44/p42), JNK, and p38, as well as an indication of PI 3-kinase activation, AKT. Both p44/42 gel shift (data not shown) and phospho-ERK 1 and 2 immunoblotting showed that ligand-dependent EMC activation rapidly stimulated ERKs 1 and 2 (Fig. 3*A*). To see this activation by EMC alone, it was necessary to withdraw IL-3 for at least 1 h because IL-3 by itself stimulates ERKs 1 and 2. The combination of EMC and IL-3 receptor signaling was, at best, minimally more effective than either ligand alone, with respect to the ERK activation. The EGF-dependent activation of ERKs 1 and 2 in EGFR-expressing cells was similar to that of EMC (data not shown). Thus, EMC can signal to the ERK pathway, but this in no way distinguishes it from EGFR or IL-3. With respect to JNK, IL-3 did not produce detectable JNK activity when added to withdrawn cells. Both EMC and EGFR signaling produced a small, very transient activation that was observed at 10 min but was nearly gone at 30 min (data not shown). AKT activation was also assessed by immunoblotting and showed that EMC signaling clearly increased phospho-AKT. IL-3 also increased phospho-AKT, but again the addition of both signals was less than additive (Fig. 3*B*). Assessment of p38 using activated p38 antibodies for immunoblotting showed some persistent p38 activation in IL-3 withdrawn cells (Fig. 3*C*). EMC activation led to a modest increase in phospho-p38 that was no greater than that of re-addition of IL-3. As with ERK and AKT, the combination of EMC and IL-3 was not substantially increased over either alone. In summary, the study of these four signaling pathways did not reveal substantial differences among EMC, EGFR, and IL-3 signaling that would explain the differences in biologic and functional output noted below.

Ligand-dependent Activation of EMC Results in an Anti-apoptotic Signal without Stimulating Proliferation—IL-3 withdrawal results in rapid death (due to apoptosis) of parental 32D cells as well as transfected cells expressing either vector alone, full-length EGFR, or EMC (*circles*, Fig. 4) with virtually all cells dying within 24–48 h. Addition of EGF upon IL-3 withdrawal had no effect on vector-transfected 32D cell lines. EGF not only prevented cell death in EGFR-expressing cells but mimicked the proliferative effect of IL-3. In EMC-expressing cells, EGF activation of the Mer signal resulted in a nearly

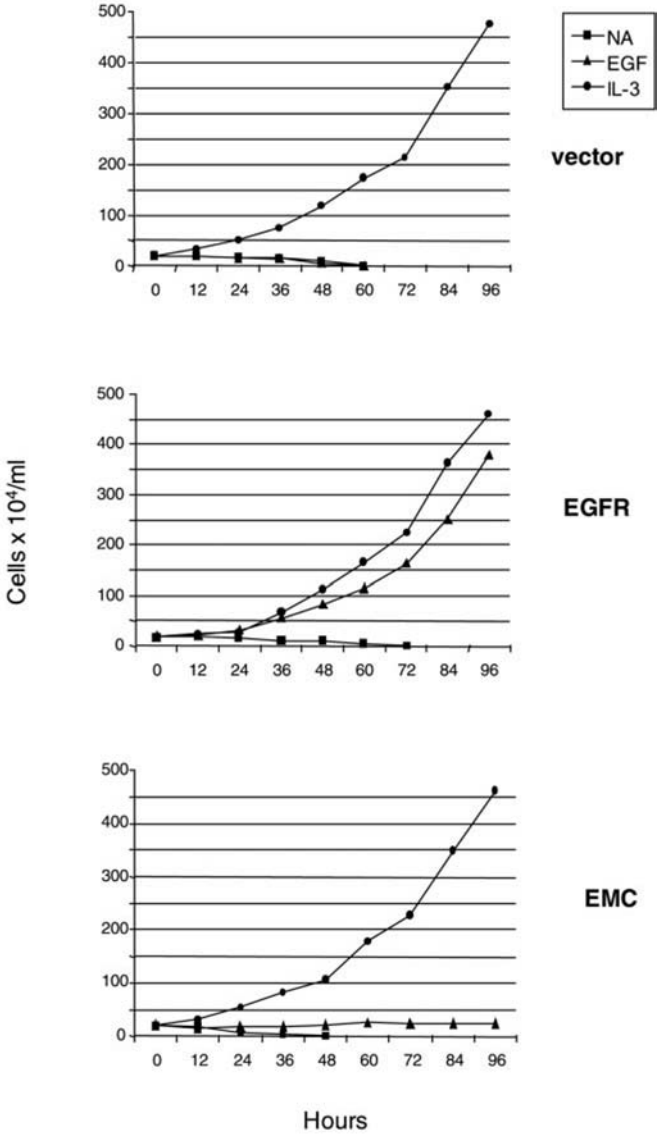

FIG. 4. **EGFR signaling stimulates 32D cell proliferation; EMC signaling does not.** 32D vector, EGFR, or EMC cells were cultured in the absence (*NA*) or presence of IL-3 (5% WEHI conditioned medium) or 100 ng/ml EGF for 4 days as described under "Materials and Methods." Viable cells were counted every 12 h through day 4. Withdrawal of IL-3 without other additions leads to apoptotic cell death. Stimulation of EGFR in EGFR-transfected cells and IL-3 in all cell lines increased cell numbers substantially. Activation of EMC resulted in a stable number of surviving cells. These data are representative of six separate experiments.

TABLE I

Kinase-inactive Mer does not protect 32D cells from IL-3 withdrawal

32D cells stably transfected with pLXSN (vector) EMC or a kinase-inactive EMC created by site-directed mutagenesis of lysine 619 to methionine 619 were treated with no additions (NA), EGF 100 ng/ml, and IL-3 (5% WEHI conditioned media). IL-3 caused proliferation in all three cell lines, but EGF only prevented cell death in the line expressing kinase active EMC.

Day	NA	EGF	IL-3
	Vector		
0	20	20	20
1	9	13	41
2	4	4	111
3	0	0	231
4			376
5			570
6			900
7			2132
	EMC		
0	20	20	20
1	13	20	34
2	0	25	83
3		27	148
4		25	412
5		23	803
6		24	1621
7		23	3352
	Kinase-inactive EMC		
0	20	20	20
1	10	12	54
2	0	0	95
3			180
4			324
5			768
6			1200
7			2521

stable cell number over 96 h (Fig. 4 and Table I). In contrast a 32D cell stably expressing a kinase-inactive tyrosine kinase EMC (K619M) failed to prevent apoptosis when treated with EGF after IL-3 withdrawal (Table I).

The stable cell number in the 32D EMC cell cultures treated with EGF could result from, at least, two mechanisms as follows: Mer activation could prevent apoptosis without stimulating proliferation or a large percentage of the 32D EMC cells might undergo apoptosis, whereas the surviving cells rapidly proliferate. To address this, vector control and EMC 32D cell lines were cultured in medium devoid of any supplements (no addition) or in medium containing IL-3 or EGF. At 24-h intervals, cells were analyzed by flow cytometry for apoptosis and necrosis after staining with annexin-V-FITC and propidium iodide (Fig. 5). By 72 h virtually all vector alone or EMC-expressing cells were stained with both annexin V and propidium iodide in the absence of any treatment (Fig. 5*A*). Conversely, in the presence of IL-3 both cell lines were >90% viable. EGF could not rescue vector alone cells (almost 100% apoptotic and necrotic) whereas >80% of the EGF-treated EMC cells were viable (Fig. 5*A*). Greater than 90% of the cells without IL-3 or EGF were dead by 48 and 72 h (Fig. 5*B*), and apoptosis was apparent within 24 h. These data (Figs. 4 and 5) indicate that the Mer signal partially replaces IL-3 action preventing apoptosis but does so without stimulating proliferation.

Mer Signaling Blocks IL-3-dependent Growth and Produces Morphologic Changes in 32D Cells—In the presence of IL-3, 32D cells grow rapidly in suspension, and in fact, the medium must be changed frequently to prevent apoptosis from overgrowth. Addition of EGF to EMC-expressing cells not only keeps them alive without proliferation but changes these suspension growing cells producing an adherent, slightly flattened phenotype (not shown). This is clearly distinguishable from the effects of persistent IL-3 or the addition of EGF in EGFR-expressing cells, both of which promote continued growth of round cell populations. A surprising result was obtained when EGF and IL-3 were added together to EMC-expressing cells. The adherent phenotype became more prominent, and IL-3-dependent growth was substantially reduced so that by 24–48 h cell numbers no longer increased. This was not seen when EGF and IL-3 were added to EGFR-expressing cells. Fig. 6 demonstrates the lack of proliferation in EGF- and IL-3-treated EMC 32D cells over a 7-day period. Vector-transfected cells, which do not express EGFR or EMC, grow in response to IL-3 or IL-3 plus EGF and die when IL-3 is withdrawn even if there is EGF in the media.

To study the state of the cell cycle, we investigated the phosphorylation of the retinoblastoma protein (Rb) and performed cell cycle analysis. Immunoblots of Rb show that addi-

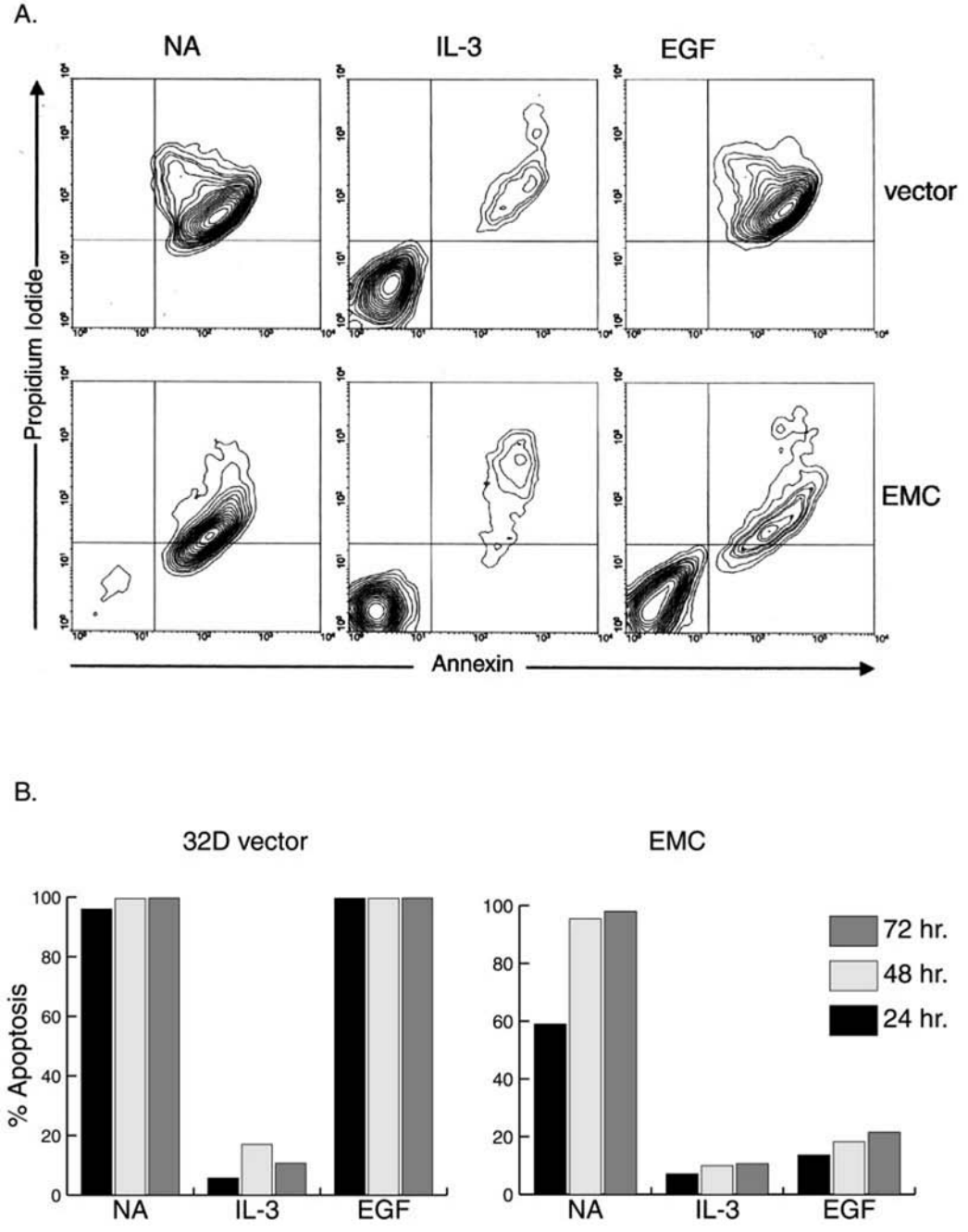

FIG. 5. **EMC signaling prevents apoptosis and flow cytometric analysis.** *A*, healthy cycling 32D vector (*top*) and EMC (*bottom*)-expressing cells were cultured in medium without additions (*NA*) or in the presence of IL-3 (5% WEHI conditioned medium) or 100 ng/ml EGF for 72 h. Cells were harvested and incubated with annexin V-FITC in a buffer containing PI and analyzed by flow cytometry. Apoptotic cells were found in quadrants labeled with both annexin V and propidium iodide. *B*, calculated percent of apoptotic cells after 24, 48, and 72 h incubation with no additions, IL-3, or EGF. Withdrawal of IL-3 led to apoptosis (annexin V-positive) and death (propidium iodide-positive). When cultured in IL-3 both cell lines continued to cycle and were largely annexin V-FITC- and propidium iodide-negative. 32D vector cells cultured in the presence of EGF were positive for annexin V and propidium iodide indicating cell death, and EMC cells cultured in EGF were ~80% viable.

tion of EGF and IL-3 led to Rb dephosphorylation as evidenced by a loss of slower mobility forms of Rb progressively from 8 to 48 h (Fig. 7*A*). Flow cytometric analysis showed that greater than 60% of 32D cells are in S phase during IL-3 treatment. Addition of EGF alone, which does not significantly stimulate growth (Fig. 4 and Table I), decreased the percentage of cells in S phase from 60 to 40% over time with the majority of cells now in the G_1 phase. The addition of EGF plus IL-3, which more rapidly blocks proliferation and changes cell shape (see below), produces a rapid decrease in the percentage of cells in S phase (to 7%) and a striking increase in the percentage in G_1 (Fig. 7*B*).

Photomicrographs of stained EMC 32D cells over the course of a 24-h treatment with IL-3 and EGF show the rapid morphologic change (Fig. 8). The morphology of typical IL-3 (or EGF-treated EGF receptor-bearing 32D cells) is shown in the 0-h frame. Within 8 h the initial flattening, caused by EGF and IL-3 addition to the EMC-containing cells, was visible, and within 12 h the dendritic-like processes that are characteristic of EMC-driven morphologic change in this clone became apparent. By 24 h the cells were adherent and dramatically different in shape. This EMC-stimulated shape change was coincident with (or proceeded) the suppression of IL-3-dependent growth, and to date we have not determined whether they are separable events, *i.e.* does the morphologic change represent differen-

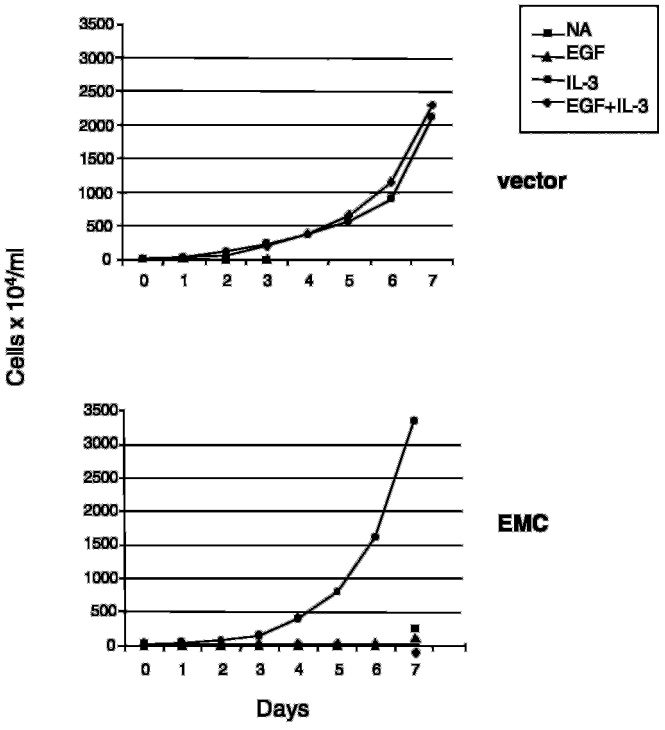

FIG. 6. **EMC stimulation prevented apoptosis and also inhibited IL-3-dependent proliferation.** 32D vector or 32D EMC-expressing cells were cultured with no additions (*NA*), 100 ng/ml EGF, IL-3 (5% WEHI conditioned medium), or both EGF and IL-3 for 7 days. Cells were counted every 24 h. IL-3 caused proliferation in both cell lines. EGF prevented apoptosis in EMC cells without substantial proliferation. Surprisingly, EMC signaling abrogated IL-3-dependent proliferation. These data are representative of three separate experiments. In the vector alone cells, all transfected cells with no additions or with EGF treatment were dead by day 3. In vector alone cells IL-3 and EGF plus IL-3 gave similar cell numbers at day 7. In the EMC-expressing cells, no addition (withdrawal of IL-3) also led to 100% cell death by day 3. With EGF or EGF plus IL-3 cell number increased from 2.0×10^5 to 2.3×10^5 and 2.2×10^5, respectively, at day 7. Addition of IL-3 alone increased cell number from 2.0×10^5 to 3.35×10^7 by day 7.

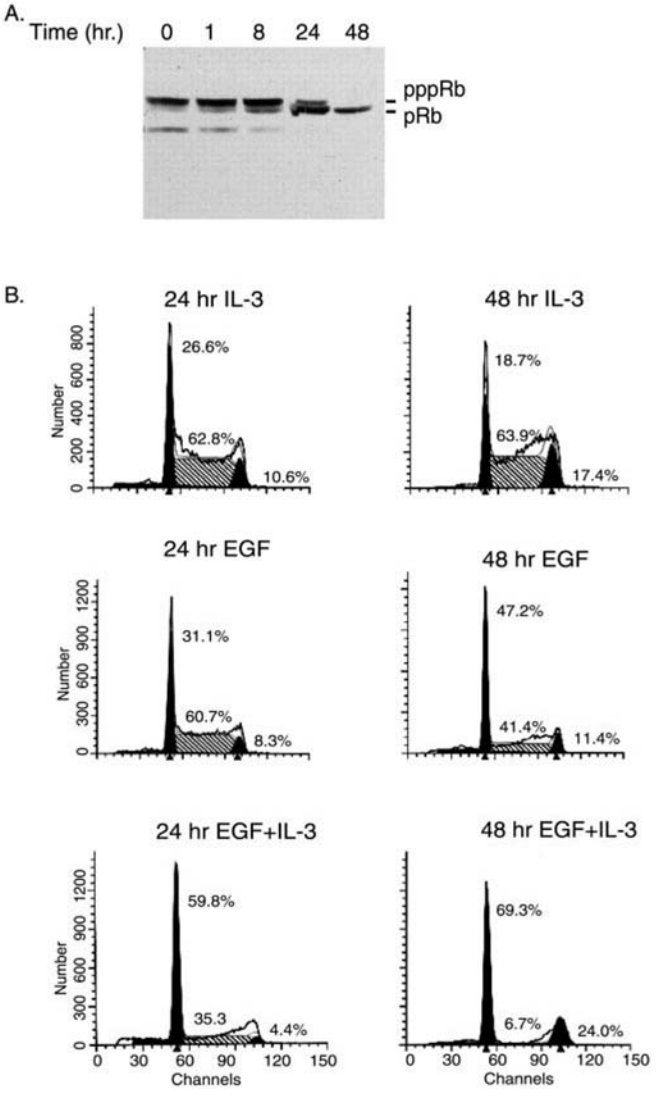

FIG. 7. **The effect of EMC and IL-3-dependent signaling on cell cycle progression.** *A*, IL-3 was with drawn from 32D EMC cells (clone A2) for 1 h. Cells were then incubated with 100 ng/ml EGF and IL-3 (5% WEHI conditioned medium) cells for 0, 1, 8, 24, or 48 h. At the indicated times cells were lysed; lysates were subjected to gel electrophoresis, followed by Rb immunoblotting. By 8 h the near uniform slow electrophoretic mobility of phosphorylated Rb protein began to change with the appearance of the lower molecular weight hypophosphorylated species. By 24 h both isoforms were detected, and by 48 h most Rb was in the hypophosphorylated state. *B*, cell cycle analysis by flow cytometry shows the effect on cell cycle stage of additions (IL-3, EGF, or IL-3 plus EGF) to 32D EMC cells at 24 and 48 h. Treatment with IL-3 maintained >60% cells in S phase. EGF activation of EMC which did not sustain proliferation over 5–7 days but which prevented apoptosis (see Fig. 4 and Table I) led to a slow decrease in S phase fraction. Combined addition of IL-3 plus EGF led to a decrease in S phase fraction at 24 h and a dramatic reduction by 48 h.

tiation that inhibits growth? Unlike EMC-expressing cells, the addition of EGF and IL-3 to EGFR-expressing cells does not alter cell morphology (not shown).

To attempt to relate the differentiated morphology, growth suppression, or anti-apoptotic signals to known signaling actions of EMC (and IL-3), we incubated EMC-expressing 32D cells in separate experiments with the PI 3-kinase inhibitor, LY294, and the MEK inhibitor, U0126. LY294 completely abolished the generation of active phospho-AKT for up to 24 h (Fig. 9*A*). Fig. 9*B* shows that U0126 completely blocked the stimulation of ERKs 1 and 2 caused by the addition of EGF and IL-3 in cells that had been withdrawn from IL-3 1 h previously. The effect of U0126 inhibition lasts at least 24 h (data not shown). Interestingly, the loss of either the ERK or the AKT pathway does *not* result in apoptosis in IL-3 or EGF-treated cells (data not shown). Thus, neither the ERK nor the PI 3-kinase pathways are necessary to prevent apoptosis under these conditions. Moreover, neither the ERK nor PI 3-kinase pathways are necessary for the dramatic morphologic change seen in EGF- and IL-3 treated cells (Fig. 9*C*). However, if both the MEK and PI 3-kinase inhibitors are added together, even IL-3- and EGF-treated cells begin to die within 24 h.

DISCUSSION

The *Axl/Ufo* receptor tyrosine kinase family consists of at least three members, each of which has several different names as follows: (i) *Axl/Ufo/Ark* (5–7); (ii) Tyro3/SKY/RSE/BRT/TIF/DTK/REK (8–13); and (iii) MER/NYK/EYK (1–4, 15). Gas6, the growth arrest-specific gene 6, binds to each mammalian member of this receptor family (16, 19–21), but the affinity for Mer is, at least, 1 log lower (10^{-9} *versus* 10^{-8} M) than it is for Axl and Tyro3 (21). The physiological consequences of one ligand binding to divergent members of a receptor family are not fully established although it is not without precedent (*e.g.* EGF receptor family ligands) (47).

The *Mer* chimera, EMC, was generated by using the extracellular and transmembrane domains of the EGF receptor, and the construction and transfection produced a tightly ligand-dependent Mer signal. This construction allowed us comparison

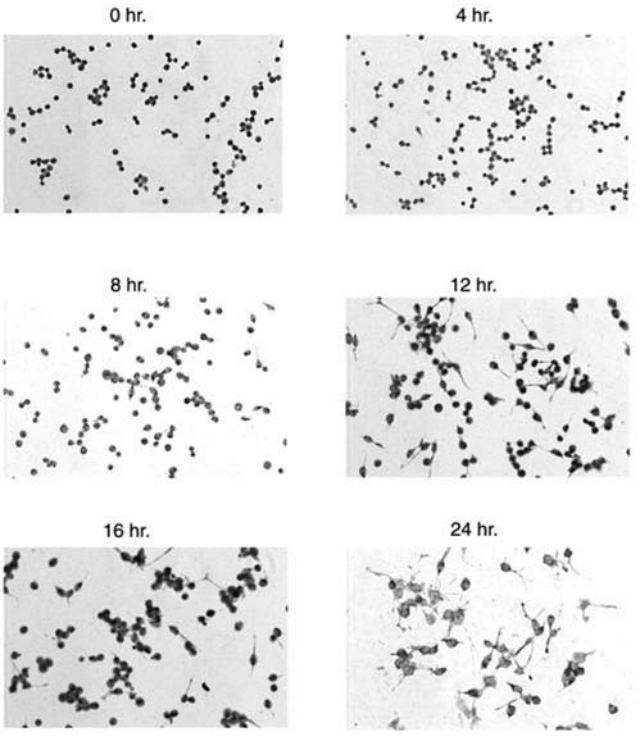

FIG. 8. **Morphologic changes in 32D EMC following stimulation with EGF IL-3.** Cells (clone A2) were treated with EGF 100 ng/ml plus IL-3 (5% WEHI conditioned medium) for the indicated times and were then fixed and stained with Wright Giemsa stain. The round cell morphology characteristic of proliferating 32D cells (*0 h*) was altered with adherence to the tissue culture dish beginning at ~8 h and by spreading and elongation of processes (12–16 h). By 24 h the majority of cells had undergone flattening and process elongation.

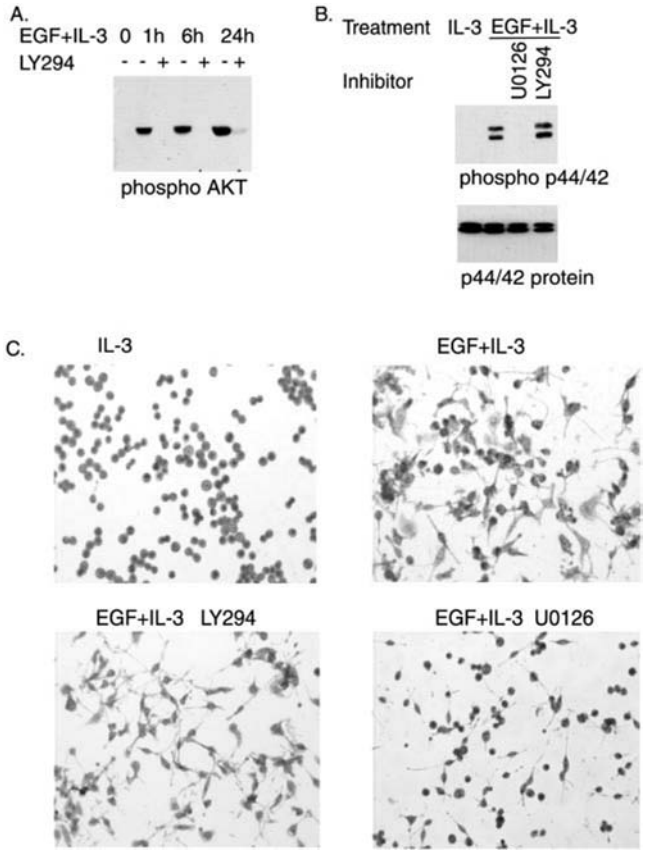

FIG. 9. **The effects of inhibiting *AKT* and ERK p44/42 on 32D cell morphology.** IL-3 was withdrawn from 32D EMC cells. They were subsequently incubated with either 10 μM LY294 (*A* and *C*) or 10 μM U0126 (*B* and *C*) for 1 h. 100 ng/ml EGF and IL-3 (5% WEHI conditioned medium) were added back. Cells were lysed at 0, 1, 6, or 24 h (*A*) or 24 h (*B*). Lysates were subjected to get electrophoresis and immunoblotted with anti-phospho-*AKT* (*A*) or anti-phospho-p44/42 or anti-p44/42 (*B*). *C*, after 24 h cells were fixed, stained with Wright Giemsa stain, and visualized. LY294 and UO126 blocked *AKT* and ERK activation, respectively, and did not change the flattened elongated morphology produced by activating EMC and the IL-3 receptor for 24 h.

of the acute Mer signal to the EGFR/*Axl* chimera made by the same method (26). Multiple receptors including HER2, EGFR, Ret, and the EGFR/*Axl* chimera all stimulate 32D cell growth and prevent apoptosis (26, 36–39), whereas EMC prevents apoptosis without stimulating proliferation. In conjunction with IL-3, the Mer signal alters morphology, dramatically, and in 32D cell clones prevents further cell growth. Clearly, the signaling output from Mer is unusual for a receptor tyrosine kinase although certain aspects of this signal may be due to its emanation from a chimera rather than wild-type Mer. Further studies defining differences between full-length and chimeric Mer signaling are needed.

Liu and co-workers (48) have shown EGF/*Axl* chimera stimulates 32D cell proliferation but that Gas6 activation of full-length Axl does not. They also showed that full-length Axl is cleaved at the surface upon the addition of Gas6 ligand to 32D cells. This change may alter signaling duration or the range of the substrates as an alternative mechanism of down-regulation is used in the Axl-bearing 32D cells as compared with those expressing the EGFR/*Axl* chimera. Whether full-length Mer and EMC have different effects remains to be determined, but it is obvious that the *Mer* chimera signal differs substantially from the many receptor tyrosine kinases that stimulate proliferation including the EGFR/*Axl* chimera.

The dramatic adhesive and morphologic changes that EMC induces in the 32D leukemic cell line derived from the monomyelocytic lineage resembles a form of differentiation. In turn, this "differentiation" could explain the cessation of IL-3-dependent growth. Prior studies of 32D cells have shown that G-CSF and IGF_1 can sustain 32D cells in the absence of IL-3 and that G-CSF and IGF-treated cells grow slowly and differentiate toward a granulocyte phenotype over 4–8 days (40–42). Thus there is similarity between the G-CSF and IGF_1 receptor differentiation pathways, both of which are distinct from that of Mer. In our 32D cell clones, G-CSF was added, and after a period of weaning from IL-3, G-CSF prevented apoptosis over 4–8 days (data not shown). Under these conditions, G-CSF-treated cells were morphologically distinct from those receiving a Mer signal. These results point to a different end point of Mer signaling compared with that of the G-CSF and the IGF_1 receptors.

In another 32D cell model system, activation of an intracellular tyrosine kinase cascade by an expressed, mutated erythropoietin receptor did result in more rapid (24–48 h) growth cessation in G_1, an effect similar to that of Mer (49). This erythropoietin receptor was constructed to activate JAK2 and leads to the tyrosine phosphorylation of STAT 3. The biologic result, which occurred in 24–48 h, was induction of the surface proteins, ICAM and CD18, and a homotypic cell adhesion phenotype resulting in clumping of cells in suspension. This clumped suspension cell phenotype is clearly different from the Mer-induced adhesive and extended process phenotype (Fig. 8). The Mer signal is thus distinct from that of G-CSF, IGF, and a mutant erythropoietin receptor.

The cellular response to Axl/Mer/Tyro3 with regard to anti-apoptotic and proliferative signaling may be cell type-specific. Substantial overexpression of Axl and Tyro3/REK can transform fibroblasts (6, 14, 24). We have not been able to transform

NIH 3T3 cells with Mer even in side-by-side transfection experiments in which Axl, using the same expression vector, was fully capable of transforming NIH 3T3 cells. Thus, the Mer and Axl signaling output upon overexpression appears to be different. Mer signaling as shown by EMC (Fig. 3) can stimulate ERK activation, and Kung and co-workers (2) demonstrated ligand-dependent (CSF1) ERK activation and proliferation in NIH 3T3 cells when they stably transfected a c-Fms extracellular domain and Mer transmembrane and cytoplasmic domain chimera into these mouse fibroblasts. Thus, a Mer signal under some circumstances can make fibroblasts grow. Hanafusa and colleagues (50) produced and stably transfected a constitutively active CD8 extracellular domain *Mer* chimera into the BaF_3 mouse lymphoid cell line. In these cells a constitutively active, ligand-independent Mer signal not only maintained survival but stimulated growth. It is interesting to note that Mer is ectopically expressed in most lymphoid leukemic cells lines (1) and in over two-thirds of childhood Acute Lymphatic Leukemia samples.[1] Perhaps *Mer* is mitogenic in lymphoid cells in which it is never expressed physiologically, whereas its function in monocytic type cells (32D cell is a myelo-monocytic cell line) is more restrained or stimulates a cytoskeletal rearrangement that leads to growth cessation. Cell type specificity is also seen in the fact that Axl in some cells can prevent apoptosis without stimulating growth (27, 28).

Recent data (30) from our own and other laboratories indicate that Mer in addition to its role in repressing immune activation may have a function in some cellular contexts in the regulation of specialized cytoskeletal rearrangement. First our group has shown that Mer plays an obligatory role in the monocyte ingestion of apoptotic material including apoptotic thymocytes induced into programmed cell death by dexamethasone (32). Particle phagocytosis proceeds normally in monocytes from *Mer*−/− mice, but although apoptotic cells bind to the Mer-deficient monocytes, they are not internalized (32). Additional genetic evidence demonstrating the role of Mer in the ingestion of apoptotic rod and cone tips by pigmented retinal epithelial cells also suggests that Mer signaling can provide a stimulus for specialized cytoskeletal control. Gene-targeted mice (30, 31), a naturally occurring rat model (34), and a human genetic disease (retinitis pigmentosa) (35) all have mutations abrogating the tyrosine kinase activity of Mer resulting in retinal degeneration. The dramatic shape changes and alteration in adhesion brought about by Mer in the 32D cell clones suggest a direct or indirect receptor-mediated signal to the cytoskeleton. The anti-apoptotic signaling may well be shared by all members of this family, but specific morphologic changes induced in at least some monocytic and epithelial cells may be more characteristic of Mer.

The intracellular signaling pathways responsible for these EMC-specific effects in 32D cells remain to be determined and must by definition differ to some extent from the EGFR *Axl* chimera and the EGF receptor. The Tyr(P) substrates for Mer differ from those of the EGF receptor (Fig. 2), and their identification may provide clues as to the mechanism by which Mer changes cell shape and stops cell cycle progression. The "usual suspects," ERKs 1 and 2, JNK, p38, and PI 3-kinase may well contribute to some of the effects of EMC but are not central to the shape changes nor do they discriminate between the EGF receptor and *Mer*. All of these pathways are activated by EMC, EGFR, and IL-3, and neither the ERKs nor PI 3-kinase by themselves appear necessary for the morphologic changes engendered by EMC (Fig. 9). One cannot even invoke the duration of activation of ERKs as a key component. Whereas the length of ERK activation appears to help distinguish the growth stimulatory effect of the EGF receptor and the differentiation effect of the nerve growth factor receptor in PC12 cells (51), IL-3 sustains ERK signaling in 32D cells without causing the morphologic changes induced by Mer. Thus, the duration of ERK activation alone cannot regulate this cytoskeletal-signaling pathway. To date we cannot separate temporally or by inhibitor experiments the "differentiation" and morphologic changes from the abrogation of IL-3-dependent growth. Until we can do so, it is cautious to suggest that the growth cessation is due to the cytoskeletal changes rather than a product of a distinct cell cycle regulatory mechanism.

Acknowledgments—We gratefully acknowledge the assistance of Ruth Dy and Debra Hunter in this work.

REFERENCES

1. Graham, D. K., Dawson, T. L., Mullaney, D. L., Snodgrass, H. R., and Earp, H. S. (1994) *Cell Growth Differ.* **5**, 647–657
2. Ling, L., and Kung, H. J. (1995) *Mol. Cell. Biol.* **15,** 6582–6592
3. Jia, R., and Hanafusa, H. (1994) *J. Biol. Chem.* **269,** 1839–1844
4. Jia, R., Mayer, B. J., Hanafusa, T., and Hanafusa, H. (1992) *J. Virol.* **66,** 5975–5987
5. Faust, M., Ebensperger, C., Schulz, A. S., Schleithoff, L., Hameister, H., Bartram, C. R., and Janssen, J. W. (1992) *Oncogene* **7,** 1287–1293
6. O'Bryan, J. P., Frye, R. A., Cogswell, P. C., Neubauer, A., Kitch, B., Prokop, C., Espinosa, R., III, Le Beau, M. M., Earp, H. S., and Liu, E. T. (1991) *Mol. Cell. Biol.* **11,** 5016–5031
7. Rescigno, J., Mansukhani, A., and Basilico, C. (1991) *Oncogene* **6,** 1909–1913
8. Lai, C., Gore, M., and Lemke, G. (1994) *Oncogene* **9,** 2567–2578
9. Ohashi, K., Mizuno, K., Kuma, K., Miyata, T., and Nakamura, T. (1994) *Oncogene* **9,** 699–705
10. Mark, M. R., Scadden, D. T., Wang, Z., Gu, Q., Goddard, A., and Godowski, P. J. (1994) *J. Biol. Chem.* **269,** 10720–10728
11. Fujimoto, J., and Yamamoto, T. (1994) *Oncogene* **9,** 693–698
12. Crosier, P. S., Lewis, P. M., Hall, L. R., Vitas, M. R., Morris, C. M., Beier, D. R., Wood, C. R., and Crosier, K. E. (1994) *Growth Factors* **11,** 125–136
13. Dai, W., Pan, H., Hassanain, H., Gupta, S. L., and Murphy, M. J., Jr. (1994) *Oncogene* **9,** 975–979
14. Biscardi, J. S., Denhez, F., Buehler, G. F., Chesnutt, D. A., Baragona, S. C., O'Bryan, J. P., Der, C. J., Fiordalisi, J. J., Fults, D. W., and Maness, P. F. (1996) *J. Biol. Chem.* **271,** 29049–29059
15. Graham, D. K., Bowman, G. W., Dawson, T. L., Stanford, W. L., Earp, H. S., and Snodgrass, H. R. (1995) *Oncogene* **10,** 2349–2359
16. Varnum, B. C., Young, C., Elliott, G., Garcia, A., Bartley, T. D., Fridell, Y. W., Hunt, R. W., Trail, G., Clogston, C., Toso, R. J., and Yanagihara, D., Bennett, L., Sylber, M., Escobar, E., Liu, E. T., and Yamane, H. K. (1995) *Nature* **373,** 623–626
17. Manfioletti, G., Brancolini, C., Avanzi, G., and Schneider, C. (1993) *Mol. Cell. Biol.* **13,** 4976–4985
18. Stitt, T. N., Conn, G., Gore, M., Lai, C., Bruno, J., Radziejewski, C., Mattsson, K., Fisher, J., Gies, D. R., and Jones, P. F. (1995) *Cell* **80,** 661–670
19. Chen, J., Carey, K., and Godowski, P. J. (1997) *Oncogene* **14,** 2033–2039
20. Mark, M. R., Chen, J., Hammonds, R. G., Sadick, M., and Godowsk, P. J. (1996) *J. Biol. Chem.* **271,** 9785–9789
21. Nagata, K., Ohashi, K., Nakano, T., Arita, H., Zong, C., Hanafusa, H., and Mizuno, K. (1996) *J. Biol. Chem.* **271,** 30022–30027
22. Evans, C. O., Young, A. N., Brown, M. R., Brat, D. J., Parks, J. S., Neish, A. S., and Oyesiku, N. M. (2001) *J. Clin. Endocrinol. Metab.* **86,** 3097–3107
23. Neubauer, A., Fiebeler, A., Graham, D. K., O'Bryan, J. P., Schmidt, C. A., Barckow, P., Serke, S., Siegert, W., Snodgrass, H. R., Huhn, D., and Liu, E. T. (1994) *Blood* **84,** 1931–1941
24. Taylor, I. C., Roy, S., Yaswen, P., Stampfer, M. R., and Varmus, H. E. (1995) *J. Biol. Chem.* **270,** 6872–6880
25. Palaszynski, E. W., and Ihle, J. N. (1984) *J. Immunol.* **132,** 1872–1878
26. McCloskey, P., Pierce, J., Koski, R. A., Varnum, B., and Liu, E. T. (1994) *Cell Growth Differ.* **5,** 1105–1117
27. Bellosta, P., Zhang, Q., Goff, S. P., and Basilico, C. (1997) *Oncogene* **15,** 2387–2397
28. Goruppi, S., Ruaro, E., and Schneider, C. (1996) *Oncogene* **12,** 471–480
29. Fridell, Y. W., Villa, J., Jr., Attar, E. C., and Liu, E. T. (1998) *J. Biol. Chem.* **273,** 7123–7126
30. Camenisch, T. D., Koller, B. H., Earp, H. S., and Matsushima, G. K. (1999) *J. Immunol.* **162,** 3498–3503
31. Lu, Q., Gore, M., Zhang, Q., Camenisch, T., Boast, S., Casagranda, F., Lai, C., Skinner, M. K., Klein, R., Matsushima, G. K., Earp, H. S., Goff, S. P., and Lemke, G. (1999) *Nature* **398,** 723–728
32. Scott, R. S., McMahon, E. J., Pop, S. M., Reap, E. A., Caricchio, R., Cohen, P. L., Earp, H. S., and Matsushima, G. K. (2001) *Nature* **411,** 207–211
33. Lu, Q., and Lemke, G. (2001) *Science* **293,** 306–311
34. D'Cruz, P. M., Yasumura, D., Weir, J., Matthes, M. T., Abderrahim, H., LaVail, M. M., and Vollrath, D. (2000) *Hum. Mol. Genet.* **9,** 645–651
35. Gal, A., Li, Y., Thompson, D. A., Weir, J., Orth, U., Jacobson, S. G., Apfelstedt-Sylla, E., and Vollrath, D. (2000) *Nat. Genet.* **26,** 270–271
36. Di Fiore, P. P., Segatto, O., Taylor, W. G., Aaronson, S. A., and Pierce, J. H. (1990) *Science* **248,** 79–83
37. Lee, J., Dull, T. J., Lax, I., Schlessinger, J., and Ullrich, A. (1989) *EMBO J.* **8,** 167–173
38. Lehvaslaiho, H., Lehtola, L., Sistonen, L., and Alitalo, K. (1989) *EMBO J.* **8,** 159–166
39. Santoro, M., Wong, W. T., Aroca, P., Santos, E., Matoskova, B., Grieco, M.,

Fusco, A., and di Fiore, P. P. (1994) *Mol. Cell. Biol.* **14,** 663–675
40. Valentinis, B., Romano, G., Peruzzi, F., Morrione, A., Prisco, M., Soddu, S., Cristofanelli, B., Sacchi, A., and Baserga, R. (1999) *J. Biol. Chem.* **274,** 12423–12430
41. Kato, J., and Sherr, C. J. (1990) *Blood* **75,** 1780–1787
42. Valtieri, M., Tweardy, D. J., Caracciolo, D., Johnson, K., Mavilio, F., Altmann, S., Santoli, D., and Rovera, G. (1987) *J. Immunol.* **138,** 3829–3835
43. McCune, B. K., and Earp, H. S. (1989) *J. Biol. Chem.* **264,** 15501–15507
44. Luetteke, N. C., Phillips, H. K., Qiu, T. H., Copeland, N. G., Earp, H. S., Jenkins, N. A., and Lee, D. C. (1994) *Genes Dev.* **8,** 399–413
45. Miller, A. D., and Rosman, G. J. (1989) *BioTechniques* **7,** 980–982, 984–986, 989–990
46. Chomczynski, P., and Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159
47. Peles, E., and Yarden, Y. (1993) *Bioessays* **15,** 815–824
48. Fridell, Y. W., Jin, Y., Quilliam, L. A., Burchert, A., McCloskey, P., Spizz, G., Varnum, B., Der, C., and Liu, E. T. (1996) *Mol. Cell. Biol.* **16,** 135–145
49. Wooten, D. K., Xie, X., Bartos, D., Busche, R. A., Longmore, G. D., and Watowich, S. S. (2000) *J. Biol. Chem.* **275,** 26566–26575
50. Georgescu, M. M., Kirsch, K. H., Shishido, T., Zong, C., and Hanafusa, H. (1999) *Mol. Cell. Biol.* **19,** 1171–1181
51. Traverse, S., Gomez, N., Paterson, H., Marshall, C., and Cohen, P. (1992) *Biochem. J.* **288,** 351–355